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Multifocus structured illumination microscopy for fast volumetric super-resolution imaging

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Abstract: We here report for the first time the synergistic implementation of structured illumination microscopy (SIM) and multifocus microscopy (MFM). This imaging modality is designed to alleviate the problem of insufficient volumetric acquisition speed in super-resolution biological imaging. SIM is a wide-field super-resolution technique that allows imaging with visible light beyond the classical diffraction limit. Employing multifocus diffractive optics we obtain simultaneous wide-field 3D imaging capability in the SIM acquisition sequence, improving volumetric acquisition speed by an order of magnitude. Imaging performance is demonstrated on biological specimens.

OCIS codes: (180.6900) Three-dimensional microscopy; (100.6640) Superresolution; (180.2520) Fluorescence microscopy; (170.2520) Fluorescence microscopy; (170.0110) Imaging systems; (180.0180) Microscopy.

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1. Introduction

Fluorescence microscopy is a powerful tool in biomedical research. Until recently, biological studies using fluorescence microscopy were restricted by the classical diffraction limit of resolution to allow the visualization of sub-cellular structures, but not their inner workings. Diffraction-limited resolution can be defined after Abbe [1] as $d = \lambda / (2 \times NA)$ where d is the finest feature in the object that can be resolved, NA the objective Numerical Aperture and λ the wavelength. Imaging green light ($\lambda \approx 525 nm$) with an objective of $NA = 1.4$ we can thus resolve structures as fine as $\approx 200 nm$. Super-resolution microscopy methods that extend the resolution of optical imaging systems beyond the diffraction limit have demonstrated great potential for new discovery in biology and medicine [2]. Biological structures that were previously only resolvable using methods that require specimen fixation (such as electron microscopy) can now be visualized in living specimens with highly specific fluorescent labeling. Super-resolution microscopy thus has potential to provide live imaging of the dynamic processes of life at the nanoscale. However, acquisition speed — especially in 3D — remains a major challenge in this field. Volumetric super-resolution acquisition rates typically range from seconds to minutes, depending on the method used [2]. In this Letter we describe an imaging modality for fast super-resolution microscopy in Biology — Multifocus Structured Illumination Microscopy (MF-SIM) — that improves volumetric acquisition speed by an order of magnitude in applications where sequential (scanning) 3D imaging rates are currently limiting. We expect MF-SIM to allow the visualization of dynamical biological processes in 3D with high contrast and extended resolution at sub-second time-scales.

2. Super-resolution imaging with structured illumination microscopy (SIM)

Structured Illumination Microscopy (SIM) is a wide-field super-resolution imaging method that is particularly well suited for biological imaging due to its gentle light-dose, excellent contrast and large field of view [3,4]. SIM works as follows: a series of wide-field images is collected under fluorescence excitation illumination by a standing wave patterns that is

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rotated and translated across the specimen. Frequency mixing between the illumination pattern and the specimen structure gives rise to dark and light fringes in the images. These fringes (analogous to Moiré fringes) contain higher-frequency information — beyond the frequency support of the objective — that is retrieved computationally to form an image with extended resolution [3]. We have here implemented linear SIM, which extends resolution by up to a factor of two beyond the diffraction limit to $d \approx 100\,nm$. Non-linear SIM has been demonstrated down to $d \approx 50\,nm$ [3,5-6] but requires the introduction of a non-linear process such as photoswitching or photobleaching.

Live 3D SIM is typically implemented with acquisition speed in the range of seconds [7,8]. At this time-scale it becomes possible to study slowly moving specimens. Bringing 3D SIM to the sub-second time-scale would greatly extend applicability in biological research, allowing visualization of fast dynamic events.

3. Multifocus microscopy (MFM) for simultaneous 3D imaging

Due to the classical conflict between spatial and temporal imaging resolution when 3D information is acquired sequentially (scanning through focus), volumetric time-lapse imaging is intrinsically time-consuming. We previously reported the method aberration-corrected multifocus microscopy (MFM) [9] which eliminates sequential focal scanning in wide-field imaging by the simultaneous formation of a 3D focal stack of 2D images. MFM 3D imaging is implemented by a specially designed diffractive optical element, made of glass, that is placed in a Fourier plane (objective pupil plane) of a wide-field microscope. The diffractive element multiplexes and focus-shifts the microscope image so as to form a 3D focal stack of 2D images. MFM thus circumvents the temporal-spatial resolution conflict and delivers 3D imaging at 2D frame rate. Spatial frequency support is absolutely maintained in the image formation process and resolution is not compromised.

Since multifocus optics operate on the fluorescence emission light-path, the technique should hypothetically be compatible with SIM, which operates by modifying the illumination light-path. A conceptual layout of an MF-SIM system is shown in Fig. 1(a). This system is designed to improve volumetric acquisition speed by providing simultaneous 3D imaging capability in the Structured Illumination acquisition sequence. Volumetric SIM image acquisition can thus be achieved at the 2D acquisition rate of the microscope, instead of at times the 2D acquisition rate, where N is the number of focal planes acquired and τ is the $(N+\tau)$ extra time required for refocusing between frames.

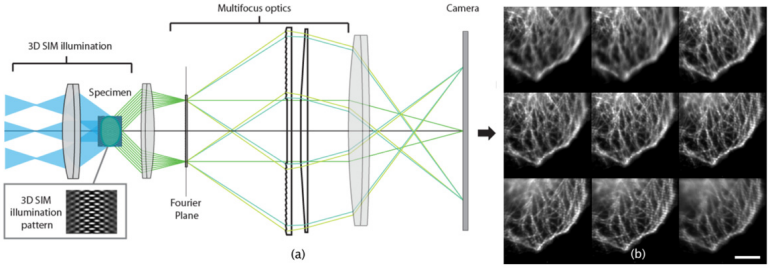

Fig. 1. (a) Conceptual layout of MF-SIM optical system. As the specimen is illuminated by the 3D SIM fluorescence excitation pattern (here in a series of 15 images: 3 directions and 5 phases), the multifocus optics form the images of nine focal planes simultaneously on the camera. Thus, instead of the $9\times15=135$ images that would be required in conventional 3D SIM, only the 15 images of the illumination sequence are required in order to cover the 3D volume. See our previous publications [9–11] for the detailed design, assembly and alignment of an MFM optical arm. (b) Fringes of SIM fluorescence excitation light create a stripe pattern across the fluorescently labeled microtubules in one of the 15 time-frames of a MF-SIM image of an U2OS cell. For a movie of the acquisition sequence, see Visualization 1. For the original data set in TIF format, see Dataset 1 [17]. Scale bar $5\mu m$.

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4. Multifocus structured illumination microscopy (MF-SIM)

We have constructed a proof of concept MF-SIM optical system, implemented as a modular add-on to the commercial SIM microscope Zeiss Elyra PS1. Hijacking the left side-port of the Elyra, we placed a lens L_1 (focal length $f=150\,mm$, diameter $\phi=25\,mm$, achromatic doublet, Newport) at focal length distance after the primary image plane. This yielded a secondary Fourier plane — an image of the objective pupil formed by the microscope's internal tube lens and the lens L_1 — where we inserted the multifocus diffractive optical element. Correction elements were placed after the primary element to remove chromatic dispersion. A second relay lens ($f=200\,mm$, $\phi=80\,mm$, achromatic doublet, Newport) was finally used to form the multifocus image on the camera. Design and manufacturing of multifocus diffractive optics has been described in detail in [10]. For a detailed description of the layout and alignment of a multifocus optical arm, see the Supplementary Material in our previous publication [11].

In the MF-SIM system here implemented, $N=9$ focal planes successively spaced $\approx 100\,nm$ nm apart cover a depth in the specimen of $\approx 1\,\mu m$ with a lateral field of view of $\approx 16\,\mu m \times 16\,\mu m$. We used a Zeiss $63\times$ oil immersion objective ($NA=1.4$) and Optovar $1.6\times$ magnification changer. Data was recorded with the Elyra standard settings: a three-beam SIM illumination pattern with 5 phases and 3 rotations. We used blue excitation light and a green 525/50nm emission filter. Images were captured on an Andor iXon-888 EMCCD camera that was synchronized with the Elyra illumination sequence.

5. MF-SIM on biological specimens

We present MF-SIM data of two well-known biological specimens. Firstly, we verify lateral resolution extension by imaging the synaptonemal complex in a mouse spermatocyte spread. Secondly, we demonstrate the capability of simultaneous 3D imaging by imaging the microtubule network in an intact U2OS cell.

Spermatocyte spreads were prepared with a drying down technique described in [12] and mounted in Prolong Gold (P36930, Life Technologies). Tubuli from decapsulated testes were dissected and fixed in paraformaldehyde. Primary antibody anti-SYCP3 (sc-74569, Santa Cruz Biotechnology), secondary antibody Alexa 488 (A-11001, Life Technologies). In this specimen, the two complex strands (lateral elements of the synaptonemal complex) of each chromosome are separated by $\approx 100\,nm$ [13] and thus not possible to resolve with classical wide-field microscopy (Fig. 2(a)). As expected, the lateral elements become clearly resolvable and can be seen twisting around each other in the reconstructed MF-SIM image (Fig. 2(b)).

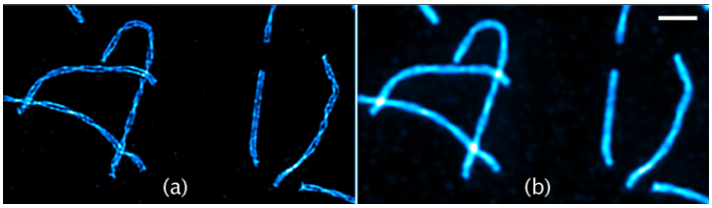

Fig. 2. Mouse spermatocyte synaptonemal complex fluorescently labeled with antibody stain for SYCP3 (Alexa 488). Complex strands of chromosomes are wrapped around each other at $\approx 100\,nm$ distance. Strands can be laterally resolved using linear SIM, but not using conventional wide-field microscopy. (a) MF-SIM image of a region of interest of a single 2D focal plane from an MF-SIM image. (b) Deconvolved wide-field image of data in *a*. See Dataset 1 and Dataset 2 (Refs. [17,18].) for raw and reconstructed images and Visualization 2 for a movie of the acquisition sequence. Data is displayed in ImageJ Cyan Hot lookup table. Scale bar. $2\,\mu m$

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Formaldehyde fixed U2OS cells were labeled and mounted in Mowiol (DABCO) as described in [14]. Primary antibody against alpha-Tubulin (T6074, Sigma-Aldrich), secondary antibody: Alexa Fluor 488 (A11001, Life Technologies). In this three-dimensional sample, we can clearly see how MF-SIM captures the volume of the cell in each image frame of the Structured Illumination sequence. Reconstructed data is displayed in Fig. 3 in multifocus configuration (Fig. 3(a)) and as a *z*-projection color-coded for depth (Fig. 3(b)).

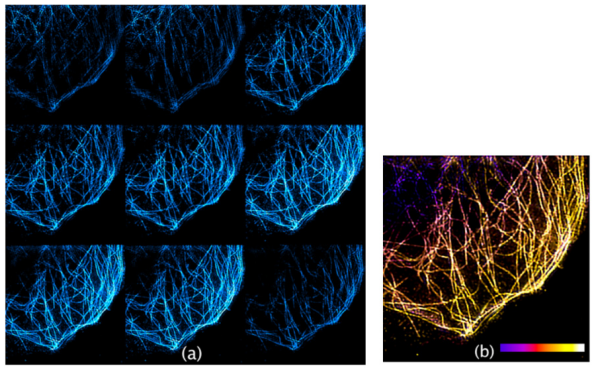

Fig. 3. Tubulin in fixed U2OS cell. (a) Reconstructed MF-SIM image frame. Nine focal planes of the MF-SIM image are seen ordered reading row-wise from left to right from top to bottom. Data is displayed in ImageJ Cyan Hot lookup table. (b) Color-coded depth projection (according to lookup table in inlay) of data in *a*. Image lateral field of view is $\approx 16\,\mu m \times 16\,\mu m$ and focal planes are separated by $\approx 100\,nm$. See Dataset 3 [19] and Dataset 4 [20] for raw and reconstructed TIF data files and Visualization 1 for a movie of the acquisition sequence.

6. Data reconstruction

Data was reconstructed using ImageJ/Fiji [15] with the free open source SIM image reconstruction plugin fairSIM [16]. Data sets were bleach-corrected using histogram matching and background was subtracted. The optical transfer function (OTF) was approximated using $NA = 1.4$ and $\lambda = 525\,nm$. Illumination parameters were estimated using fairSIM default settings and confirmed empirically. OTF attenuation (strength 0.98, FWHM 1.2) was used for suppression of out-of-focus information. Wiener parameter was set to 0.05 and apodization cutoff to 2. For image display, negative intensity values were truncated and images were contrast adjusted. Each focal plane was cropped out and processed independently. To assemble the 3D stack, focal planes were aligned with the StackReg plugin. (Reconstruction results were also verified with the proprietary software Zen on the Zeiss Elyra.) Raw and reconstructed image files are available for download in Dataset 1, Dataset 2, Dataset 3, and Dataset 4 (Refs [17–20].).

It should be noted that MF-SIM data is here not recorded by translating the specimen through the illumination pattern as in classical 3D-SIM. Instead, the specimen is kept stationary and the shift of the Structured Illumination pattern creates the modulation (See Visualization 1 and Visualization 2). Therefore, the classical 3D-SIM reconstruction scheme [2] is not applicable. We have here opted to register and reconstruct each focal plane separately using the 2D-SIM image reconstruction algorithm slice by slice. This method provides proper lateral resolution extension and some rejection of out-of-focus light, but not the axially extended resolution that can be obtained by the 3D-SIM algorithm. We expect that it will be possible to find reconstruction schemes for MF-SIM data that deliver both axial resolution extension and true optical sectioning capability, since there is modulation in each 3D point of the specimen. Optimized illumination strategies may further increase acquisition speed and simplify data reconstruction.

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7. Discussion

In summary, we have here demonstrated a volumetric super-resolution microscopy methodology — MF-SIM — and applied it in biological imaging. Although here not demonstrated on living specimens, MF-SIM is intended for applications in live super-resolution microscopy where it could allow the visualization of fast dynamic biological processes. To reach this regime in living specimens, several parameters must be optimized: Firstly, multifocus imaging is limited to the 2D acquisition speed of the microscope into which it is incorporated. Recording a single 15-frame SIM image acquisition sequence (3 rotations and 5 translations of the Structured Illumination excitation light pattern) at the fastest setting of the Zeiss Elyra intrinsically takes over one second. Thus, our proof of concept system does not have optimal acquisition speed. For implementation of MF-SIM in live-cell imaging, the concept should in the future be implemented on a high-speed SIM system. Presently, high-speed SIM systems can generate the Structured Illumination acquisition sequence with 2D frame rates fast enough for live imaging — 10 Hz with the DeltaVision OMX and even faster in custom, home-built systems. Thus implemented, we expect MF-SIM to have extensive impact on biomedical research by bringing temporal resolution of volumetric super-resolution imaging to the sub-second timescale. Signal strength is another important consideration in live imaging applications. Analogously to the classical, scanning focal stack, each additional focal plane in the multifocus image comes with increased demand on the photon budget. Fluorescence emission light is multiplexed and distributed between the N focal planes. Either excitation illumination strength or exposure time must be increased compared to the 2D case. Since the goal of MF-SIM is to optimize acquisition speed, it is preferable to increase the illumination intensity rather than the exposure time in order to obtain sufficient signal at the fastest frame-rate of which the hardware is capable. Finally, MF-SIM could conceptually be implemented with *e.g.* $N = 7$ or $N = 25$ simultaneously acquired focal planes [11] — instead of the $N = 9$ planes here demonstrated — to optimize for the thickness and photon budget of the specimen at hand.

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Disclosures

The authors declare that there are no conflicts of interest related to this article.